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Original Article

Molecular characterization of clinical isolates of *Cyclospora cayetanensis* from patients with diarrhea in India

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Abstract

Purpose: *Cyclospora cayetanensis* is an intestinal coccidian protozoan that has emerged as an important cause of both epidemic and endemic protracted diarrhea worldwide. Though humans appear to be the only natural hosts; the role of animals as natural reservoir is uncertain but of increasing concern. The present study aimed to study the prevalence of coccidian in different groups such as immunocompromised, clinically apparent immunocompetent and healthy individuals. Also, the study isolates were assessed for heterogeneity among the sequences. **Materials and Methods:** Stool samples from different groups of patients were collected. The parasite was detected in stool by different diagnostic tools such as light microscopy and nested PCR-restriction fragment length polymorphism using 18S ribosomal RNA as the target gene. **Results:** The prevalence of *C. cayetanensis* was 2.4% (19/800) in the present study. The PCR assay amplified *Cyclospora cayetanensis* DNA in only 89% (17/19) isolates. Further, sequencing revealed no significant difference among the study isolates and the non-primates. Phylogenetic analysis of the study isolates however, formed two clusters. While one cluster showed close evolutionary association with the *C. cayetanensis* strains, the other cluster showed evolutionary association with the two non-primate species. **Conclusion:** The methods described here for detection of *C. cayetanensis* oocysts are simple, efficient, specific, and sensitive and therefore can be effectively applied for laboratory diagnosis and environmental assessment of fresh produce and water sources. Clinicians should include Cyclospora infection in the differential diagnosis of prolonged or relapsing diarrheal illness even in clinically apparent immunocompetent individuals.

Key words: *Diarrhea, prevalence, phylogenetic analysis*

Introduction

Cyclospora cayetanensis formerly known as cyanobacterium-like body (CLB) is closely related to the genus Eimeria.[1] It is an obligate intracellular, sporulating coccidian protozoan parasite that infects epithelial cells of the upper small intestine, usually the jejunum. Cyclosporiasis appears to be the more common disease of tropical and sub-tropical countries and one of the causes of traveller's diarrhea. Humans appear to be the only known natural host of this parasite. It has been recognized worldwide as an emerging pathogen in both immunocompromised and immunocompetent individuals.[2,3] The most common symptom is watery diarrhea which can be severe especially in immunocompromised individuals. In some cases low grade fever and evidence of malabsorption of D-xylose may be present.[4] Infection responds to treatment with trimethoprim and sulfamethoxazole, whereas, untreated persons can have remitting relapsing illness for several weeks and months.[5]

The oocysts are shed unsporulated with the feces of infected individuals and can take from one to several weeks to become fully sporulated and infectious.[6] Hence, person to person transmission is unlikely. Humans become infected by ingesting mature oocysts through contaminated food and water.[7] Transmission of oocysts through soil, domestic animals and arthropod vectors has also been reported.[8]

The genus *Cyclospora* was first named by Schneider (1881) who recognised and identified *C. glomericola* in millipedes.[9] Since this time, over 19 recognised species have been described in reptiles, mostly snakes, insectivores, rodents and primates, including humans.[9] Of these *Cyclospora* species, sequence data are available for only 4: *C. cayetanensis* from humans, *C. cercopitheci* from

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green monkeys, *C. colobi* from colobus monkeys and *C. papionis* from baboons using *18S small subunit rDNA*, *5.8S* internal transcribed spacer regions *ITS1* and *ITS2* as the target genes.[10] These four species have a high degree of homology in the nested *18S rRNA* gene PCR assay and further have no differences in the sequences in the *Mnl*I restriction region; hence, the four species appear identical by PCR-RFLP analysis.

Molecular detection of *Cyclospora* from food products, environmental samples and clinical specimens has relied on a nested-PCR assay that amplifies a 294-bp fragment of the *18S rRNA* gene. However, the same size product is also amplified from genus *Eimeria*. Restriction fragment length polymorphism (RFLP) analysis is based on limited sequence heterogeneity within the 294-bp amplified region and is therefore, useful in differential identification of *C. cayetanensis* and *Eimeria* species.[11]

The epidemiological features of human cyclosporiasis is poorly understood in the developing countries, which further delineates the need to study risk factors and route of spread of the infection, in order to institute proper control and preventive measures. Considering the availability of almost negligible data about molecular characterization of cyclosporiasis in India, the present study therefore, aims to explore the current epidemiological knowledge by molecular typing of *Cyclospora cayetanensis* by PCR-RFLP using *18S rRNA* as the target gene in patients with diarrhea.

Materials and Methods

During the study period of three years, we received fecal samples from 600 patients with diarrhea (acute, persistent and chronic) and 200 healthy controls without any gastrointestinal complaints. Of the total, 300 were immunocompromised patients (200 adults, 100 children). *Cyclospora cayetanensis* oocysts were detected in 19 of the total patients comprising of 6 immunocompromised individuals by light microscopy. This included 16 adults and 3 children (below 12 years of age).

Ethical approval

The necessary ethical clearance was obtained from institutional ethics committee to conduct the study.

Microscopic identification of C. cayetanensis

The presence of *C. cayetanensis*, was confirmed by non-refractile spheres containing a cluster of refractile, membrane-bound globules on wet-mount examinations from the concentrated stool specimen and variably acid-fast spheres when stained with modified kinyoun's stain measuring 8–10 µm in diameter. All the three consecutive samples obtained from each patient were examined by light microscopy, however, for molecular analysis, all the three samples were pooled and considered as one sample per patient.

DNA extraction

About 220 mg of fecal sample was treated with 8–10 glass beads (0.5 mm) for oocyst disruption and genomic DNA was extracted using QIAamp kit (QIAGEN Inc., Valencia, CA) as per the manufacturer's protocol with some modifications such as the incubation time of lysis at 95ºC was increased from 10–30 minutes. The extracted DNA was stored at −20ºC till further use.

PCR amplification

PCR amplification was performed using *18S rRNA* as the target gene in a nested PCR assay using oligonucleotide primers F1E (5'- TACCCAATGAAAACAGTT-3') as forward primer and R2B (5'- CAGGAGAAGCCAAGGTAG-3') as the reverse primer for the external round of PCR. This pair amplified a primary amplicon of 636 bp. The inner primer pair F3E (5'- CCTTCCGCGCTTCGCTGCGT-3') and R4B (5'- CGTCTTCAAACCCCCTACTG-3') as the forward and reverse primer, respectively generated a 294 bp amplicon.

The 25 µl PCR reaction was carried out using 1X buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM primers and 2U Taq DNA polymerase. The PCR protocol consisted of initial denaturation at 94°C for 5 minutes followed by 35 cycles of denaturation at 94ºC for 30 seconds, annealing at 53ºC for 30 seconds, extension at 72ºC for 90 seconds and a final extension at 72ºC for 10 minutes in an ABI 2720 thermocycler (ABI Biosystems, USA). PCR condition for the nested round was same as the external round except for annealing temperature being 61ºC.[12] The product was analyzed by electrophoresis on 1.5% agarose gel stained with ethidium bromide and was visualized on a UV transilluminator.

RFLP analysis

20 µl of the nested PCR product obtained by using conventional primers was digested with 2U of the restriction endonuclease *Mnl*I (New England Biolabs, UK) in a 25 µl reaction volume for 2h at 37°C.[11,12] The digested products were fractionated on a 2% agarose gel containing ethidium bromide (0.2 µg/ml) and visualized on a UV transilluminator.

Sensitivity and specificity of PCR assay

Specificity of the PCR assay was determined by amplifying DNA from *Campylobacter jejunii*, and intestinal protozoans including *Giardia lamblia*, *Cryptosporidium* species and *Isospora belli* using the primers for *Cyclospora cayetanensis*. Sensitivity was determined by extracting

DNA from a sample with large number of oocysts (13 per OIF) followed by serial dilution of the DNA to calculate proportional quantity of DNA added to the PCR.

Sequencing

Following electrophoresis, the PCR products were excised and purified using MinElute Gel Extraction Kit (Qiagen) according to the manufacturer's instructions and sequenced on both strands (by Chromous Biotech, Germany). Chromatograms and sequences were analyzed with reference sequences obtained from the GenBank database with the following accession numbers: *C. cayetanensis*, AF069561; *C. cercopitheci*, AF111184; and *C. colobi*, AF111186 using Clustal W software after manual editing of the alignments using the BioEdit program version 7.0.9.0.

The evolutionary history was inferred using the Neighbour-Joining method.[13] The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.[14] The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method[15] and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA5.[16]

Results

The overall prevalence of *C. cayetanensis* was 2.4% (19 of 800 cases). Cyclospora infection was more common in clinically apparent immunocompetent patients with diarrhea (4.3%, 13 of 300 cases) than the immunocompromised group (2%, 6 of 300 cases). The immunocompromised group with cyclospora comprised of HIV seropositive patients (2%, 4 of 186) and post renal transplant cases (14%, 2 of 14). No cyclospora was detected in the control group. In the present study, adults (16 of 19) were more commonly infected than children (3 of 19). *C. cayetanensis* predominantly infected adults (12 of 16) and immunocompromised children (2 of 3). Further, males (18 of 19) outnumbered the females in harboring the parasite.

The most common clinical presentation of *Cyclospora* infection in the present work was prolonged watery diarrhea, abdominal cramps, distension, vomiting, fever, loss of appetite and unintentional weight loss. There was no significant difference in clinical features among patients with diarrhea and the immunocompromised group [Table 1]. However, in the present study CD4 count was less than <200 cells/µl in two of the three patients. 6 of the 19 (32%) patients were co-infected with some pathogenic or non-pathogenic parasite. Three patients (1 immunocompromised) were co-infected with *G. lamblia* and 1 HIV patient was co-infected with *Isospora belli*.

The *18S rRNA* gene PCR assay was performed for all the 600 cases and 200 controls. The PCR assay amplified *Cyclospora cayetanensis* DNA in only 89% (17/19) isolates. No *Cyclospora* was detected in the microscopically negative cases and controls. The 2 specimens negative by PCR but positive by microscopy showed either few or moderate (5-10 oocysts per smear) numbers of *Cyclospora* oocysts. This indicates that the sensitivity of PCR is 89% and specificity is 100%. The primers for *Cyclospora cayetanensis* showed no cross-reactivity when tested against *Campylobacter jejunii*, and intestinal protozoans including *Giardia lamblia*, *Cryptosporidium* species and *Isospora belli*. Sensitivity of nested PCR assay was also calculated by making 10 fold serial dilutions of the extracted DNA. The PCR amplified DNA at dilution of 10^{-6} [Figure 1].

To distinguish between *Cyclospora* and *Eimeria* spp., RFLP analysis was subsequently performed on all PCR amplicons using the restriction endonuclease *Mnl*I. As shown in Figure 2, the resulting banding pattern corresponding to 140, 106 and 48 bp was consistent with the presence of *Cyclospora* spp. in all the samples analyzed by PCR. Sequencing was performed for representative 7 isolates comprising of two immunocompromised patients. The sequences obtained showed homogeneity with the reference sequences [Figure 3]. Also, no significant difference was observed among the amino acid changes between the study isolates and the non-primates. However, phylogenetically they were different and formed two clusters. The isolates CC2, CC5, CC6 and CC7 were clustered in one group and had close evolutionary association with the *C. cayetanensis* strains. The second cluster was formed by the study isolates CC1, CC3 and CC4 along with the *C. cayetanensis* reference strain and the two non-primate species. However, the isolate CC4 was phylogenetically divergent from non-human primate species by 67 bootstrap values [Figure 4].

Demographic variables such as type of residence, availability of sanitation, source of water supply and presence of animals or pets in the household were analyzed to identify potential source of infection. However, none of the variables were significant in either of the two groups [Table 2].

Discussion

Cyclospora cayetanensis is endemic in India.[8] The prevalence of *Cyclospora cayetanensis* is higher in developing countries than in Europe and North America.[17] The present study findings revealed 19 clinical subjects positive for non-refractile spherical organisms measuring 8-10 µm in diameter. The cysts exhibited variable acid fastness consistent with *Cyclospora* species. The prevalence rates are generally higher in immunocompromised compared to immunocompetent patients.[17,18] However, the rate of *Cyclospora* infection was higher in clinically apparent immunocompetent

Table 1: Clinical symptoms among 19 patients with *Cyclospora* infection

Symptoms	Clinically Apparent immuno-competent No. (%) (*n*=11 of 13)	Immuno-compromised No. (%) (*n*=5 of 6)	Total No. (%) (*n*=16 of 19)	Statistical analysis
Duration of diarrhea (median, range)	3.0 (2, 24)	3.0 (0.5, 7.0)	3.0 (0.5, 24)	-
Abdominal pain	7 (64%)	4 (80%)	11 (69%)	1.00
Distension	7 (64%)	1 (20%)	8 (50%)	0.28
Vomiting	2 (18%)	2 (40%)	4 (25%)	0.55
Fever	1 (9%)	3 (60%)	4 (25%)	0.06
Loss of appetite	2 (18%)	5 (100%)	7 (44%)	0.005
Weight loss	7 (64%)	5 (100%)	12 (75%)	0.25
Anemia (median, range) (Hb<12.0 gm/dl)	12.2 (8.7, 14.1)	11.1 (8.3, 13.2)	12.0 (8.3, 14.1)	0.39

Hb= Haemoglobin

Table 2: Demographic factors among 19 patients with *Cyclospora* infection

Variable	Clinically apparent immunocompetent patients No. (%) (*n*=10/13)	Immunocompromised patients No. (%) (*n*=4/6)	Total No. (%) (*n*=16/19)	Statistical analysis
Residence				
Urban	7 (70)	1 (25)	8 (57)	0.25
Rural	3 (30)	3 (75)	6 (43)	
Type of house				
Pucca	9 (90)	3 (75)	12 (86)	0.51
Kuchha	1 (10)	1 (25)	2 (14)	
Water source*				
Tap water	6 (55)	2 (50)	8 (53)	0.32
Handpump	3 (27)	1 (25)	4 (27)	
Tubewell	2 (18)	0	2 (13)	
Spring water	0	1 (25)	1 (7)	
Filtered water*	7 (64)	2 (50)	9 (60)	1.00
Unfiltered water	4 (37)	2 (50)	6 (40)	
Sanitation facility in house	10 (100)	3 (75)	13 (93)	0.29
Cattle/pets	0	3 (75)	3 (20)	0.01

Variables marked (*) were analyzed for 11/13 clinically apparent immunocompetent and 4/6 immunocompromised patients

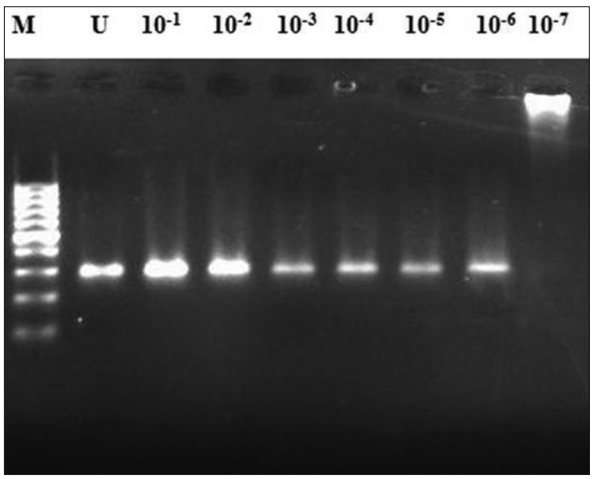

Figure 1: Lane 1: 100 bp molecular marker (Fermentas), Lane 2: Undiluted (U) nested PCR product, Lane 3-9: 10 fold serial dilutions of the nested PCR product

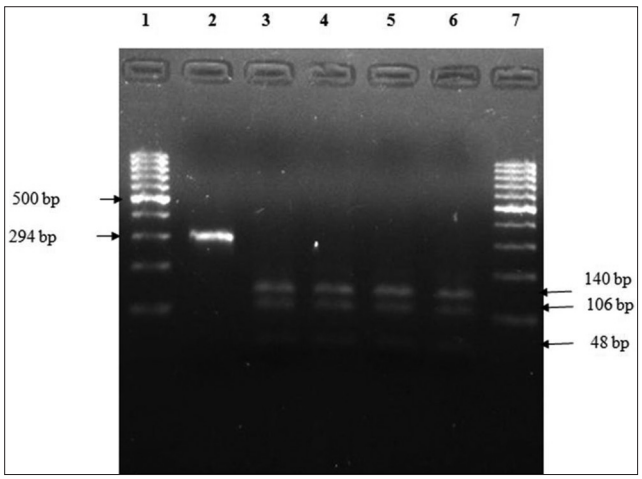

Figure 2: Lane 1, 7–100 bp molecular marker (Fermentas), Lane 2- un-restricted PCR product (*C. cayetanensis*), Lane 3,4,5,6- *MnlI* digested isolates of *C. cayetanensis*

than immunocompromised patients in our study. Additionally, these patients harbored more parasites than the immunocompromised patients. To the best of our knowledge, this is the first report showing high prevalence

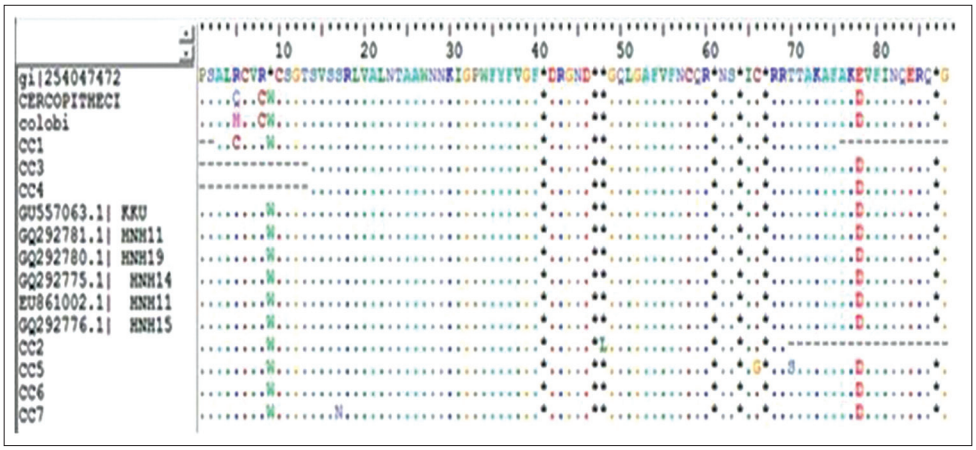

Figure 3: CC1-CC7 are study isolates, gi | 254047472- *C. cayetanensis* reference

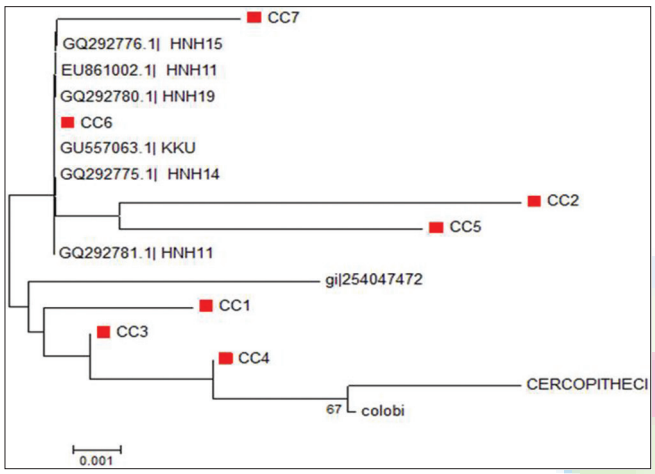

Figure 4: KKU, HNH11- HNH19 and 254047472 are *C. cayetanensis* reference sequences; CC1-CC7 are the study isolates

in the clinically apparent immunocompetent and in post renal transplant patients.

There are reports that suggest no sexual predilection for cyclosporiasis.[8,17] However, in a study from South India, 22.9% (8/35) males were infected with *Cyclospora* than 26.7% (4/15) females among the HIV patients.[19] Ours is the second study from India reporting the prevalence of the *Cyclospora* infections to be more in males (4.5%, 18 of 393) than in females (0.5%, 1 of 207). The maximum parasitic isolation in HIV patients is when CD4 counts are <200 cells/microliters.[20] In this study, CD4 counts <200 cells/microlitres were present in two of the three HIV patients.

Extraction of high-quality DNA from coccidian oocysts is challenging due to their tough outer wall, which is resistant to both chemical and physical lysis. Many other factors such as age, strain, storage conditions of oocysts, nature (viscous, turbid) of stool samples, presence of inhibitors may also impact the effectiveness of DNA extraction.[6] Failure to detect *Cyclospora* in the two microscopically positive samples could be due to any of the above mentioned factors or shearing of DNA during extraction. Hence, a more desirable approach for molecular detection of *Cyclospora* lies in efficiently recovering DNA from the oocysts present in sample matrices which contain PCR inhibitors.

The current method to detect the parasite uses a nested PCR assay that amplifies a 294 bp region of the *18S ribosomal RNA* gene, followed by RFLP and sequence analysis. Since the amplicons generated from *C. cayetanensis* and *Eimeria* species are the same size, an additional procedure like RFLP analysis is required to distinguish between the two species. The current PCR-RFLP protocol, however, cannot distinguish between *C. cayetanensis* and the other non-primate species of *Cyclospora*, hence the need of sequencing. A better approach however, would be to design primers that identify single nucleotide polymorphisms (SNPs), especially at the 3' end of the primers. These SNP primers have the potential to differentiate between *C. cayetanensis*, non-human primate species of *Cyclospora* and *Eimeria* species.[21] The PCR assay for *18S rRNA* gene was highly sensitive and specific and was also capable of overcoming many limitations of microscopic diagnosis. Further, the primers used in the PCR assay can be used for analyses of food or environmental sources suspected of harbouring these parasitic pathogens.

This paper reinforces the fact that *Cyclospora cayetanensis* is the only species associated with human illness. The mode of transmission in humans is not from non-human or primate derived *Cyclospora* spp. but through exposure to faecally contaminated food, water or soil. Further, the study also reports the prevalence of cyclosporiasis in clinically apparent immunocompmetent and immunocompromised patients in our part of the country. Also an attempt has been made to understand the risk factors for the infection for adopting adequate measures for risk management and prevention of transmission.

Many questions remain unanswered about the epidemiology of this emerging protozoal parasite. Although, the role of nonhuman primate *Cyclospora* species in causing human illness is not clear, their presence in the environment is nonetheless a public health concern.[22] Their potential as contaminants of water and food sources underscores the importance of an accurate and rapid identification for timely diagnosis. Cyclosporiasis though an enigma, thus presents an ever broadening frontier for multiple disciplines of medicine.

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